NOAA TECHNICAL MEMORANDUM

NMFS-SEFSC-283

A UNIVERSAL METHOD FOR PREPARING, SECTIONING, AND POLISHING FISH OTOLITHS FOR DAILY AGEING

by

Sheryan P. Epperly, Dean W. Ahrenholz and Patricia A. Tester

April 1991

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INTRODUCTION

The techniques for using otoliths for annual age determinations in fish are well established (Williams and Bedford 1974). The roughly seasonally alternating pattern of opaque material deposition during fast growth and hyaline material deposition during slow growth, allows researchers to assign an annual age to the fish. Pannella (1971, 1974) discovered finer increments in the otoliths and suggested they represented the daily growth. Brothers et al. (1976) later confirmed the daily periodicity of the increments and using SEM showed that these increments were the smallest cyclical units found in the otolith of Engraulis mordax. These increments are composed of incremental and discontinuous units. The discontinuous unit is relatively less calcified and is mostly composed of the protein otolin while the incremental unit is mostly composed of aragonite (Campana and Neilson 1985).

Daily growth increments have been used predominately to age larval fishes. Application of the technique to older fishes has been difficult because of problems incurred when working with larger otoliths. First, it is difficult to interpret the counts made on adult fish because it is unlikely that increment growth is daily in older fish (Ralston and Miyamoto 1983). Second, unlike otoliths of larvae which can be viewed whole, larger otoliths require time-consuming processing to achieve a viewing plane where all increments are visible. This has been accomplished by polishing the whole otolith, generally in a sagittal plane (Brothers et al. 1976, Boehlert and Yoklavich 1985, Rice et al. 1985, Neilson and Geen 1986, Gutièrrez and Morales-Nin 1986), by selective etching (Methot 1983), or by clearing the whole otolith (Struhsaker and Uchiyama 1976, Ralston 1976). Few researchers have sectioned otoliths (Ralston and Miyamoto 1983) or polished in a plane other than sagittal (Struhsaker and Uchiyama 1976, Taubert and Coble 1977, Watabe et al. 1982, Fowler 1989).

There are numerous advantages and disadvantages to these polishing or sectioning techniques. Polishing by hand has the disadvantage of uneven polishing and lack of precision in repeating the plane. Neilson and Geen (1981) developed a methodology to eliminate these problems by using a grinding jig. Sectioning has the advantage of simultaneously creating two parallel surfaces on a relatively thin section (<0.5 mm) encompassing the primordia, repeatably on the same plane. It also gives the option of selecting the plane of the cut. Because increment growth is not symmetric in otoliths and the axis of growth changes with age

(Williams and Bedford 1974), it is critical to choose a plane where 1) all increments are visible and, 2) increment spacing is the most proportional to somatic growth. The otoliths of many species are concave on the distal surface. This curvature in the growth axis makes it nearly impossible to satisfy 1) above when polishing a large otolith in the sagittal plane.

We have developed a methodology that allows thin sections to be made on **any size** otoliths in any plane and describe it in detail in the following pages. Briefly, the otolith is embedded in polyester resin. After curing, the embedded otolith is precisely aligned and mounted on a glass slide and sectioned with a low speed saw. The section is removed, attached to another slide with thermoplastic resin, and polished by hand using a descending series of abrasive papers. When the primordia is reached, if needed, the section is heated, turned and polished from the opposite side. Etching with HCl or EDTA is helpful for light microscopy and mandatory for SEM.

ACKNOWLEDGMENTS

We would like to thank John Mark Dean for welcoming us into his lab to learn his preparation methods. The use of bleach to dissolve cranial tissue and clean the otoliths was learned there. Thanks to Beatriz Morales-Nin who suggested controlling the temperature of EDTA as a solution to our inconsistent etching results. She was right. We also appreciate the many helpful and encouraging comments of Cynthia Jones and the constructive reviews of John Merriner, Allyn Powell and Dave Peters.

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DESCRIPTION OF METHOD

The following materials are needed throughout:

Consumables	Equipment
distilled water	stereo microscope
disposable pipettes and bulb	compound microscope
tissues	fine forceps
	fine probes

CLEANING OTOLITHS

Consumables	Equipment
cell tissue culture plates	fume hood
bleach (5% Na hypochlorite)	

Otoliths must be cleaned of attached tissue before processing. Depending on the size of fish, place head, section of head, or otolithic sacs in labelled wells of tissue culture plates. In a well ventilated area, fill wells with a 5% sodium hypochlorite solution (bleach) and allow to remain under a hood for at least 12 hours but not more than 24 hours. Pipet off solution and fill wells with distilled water. If tissue is dissolved and otoliths are clean, rinse otoliths a few hours to overnight. Otherwise, pipet off the water and fill the wells with the sodium hypochlorite solution a second time. Repeat rinse procedure until the otoliths have been rinsed with distilled water at least twice. It may be necessary to move otoliths to a clean tray. Pipet off all water and store dry.

EMBEDDING OTOLITHS

Consumables	Equipment
aerosal can of silicone spray	embedding mold
20 ml disposable beaker	2 micropipets
clear polyester resin	fume hood
hardener	
disposable micropipet tips	
gloves	

Otoliths are embedded for handling, orientation, and support during sectioning. Epoxy or other suitable resins can be substituted for polyester resin, but proportions and handling may vary.

Spray a clean embedding mold with a silicon spray. Set aside to dry. Under a fume hood, mix 6 ml of clear polyester resin with 50 µl of hardener in a 20 ml disposable beaker and stir briefly but well. Pipet the resin mixture into the molds, filling each over half full. Allow to set overnight. (Used pipette tips, beaker, and tissues should be left under the hood to allow resin on them to harden, then dispose of them.)

Place otolith on top of hardened resin, forward of center. Wick away excess water with a tissue. Orient otolith with its anterior-posterior axis perpendicular to the long axis of the mold if a frontal section is desired, or if a transverse section is desired, orient the otolith parallel to the long axis of the mold. A stereo microscope is usually needed to orient the otolith, hence a transparent mold is of advantage in accommodating transmitted light.

Mix resin and hardener and gently pipet the mixture over the otoliths, overfilling the molds until the surface is well-domed. The resin will shrink while curing. Allow to set overnight.

SCORING SLIDES

Consumables	Equipment
25 x 75 mm microscope slides	low speed saw
water-insoluble ink	2 diamond wafer blades
dishwashing detergent	blade spacer(s)
single-edge razor blades	

Slides are scored so that the specimen can be precisely aligned on the slide and the slide can be realigned with the blades.

Prepare saw by filling coolant reservoir with water and a couple of drops of dishwashing detergent. Collate blade assembly by assembling in sequence: 1 3/4 inch flange, blade, spacers totalling 0.5 mm in thickness, blade, 1 3/4 inch flange and mount blades on saw motor spindle.

Place microscope slide in the long saddle chuck so that the 25 mm edge is parallel to arm. Add 25 g weight to arm and start saw, setting rheostat between 6 and 7. Lower arm gently, allowing slide to touch blades and be scored briefly. Repeat for each slide, as needed.

Clean and dry slides. Place slide, scored side up, on a paper towel and place a drop of water-insoluble ink on the scored area. Allow ink to dry, then scrape the ink off the surface of the slide with a single-edge razor blade. Ink will remain in the scored lines on the slide.

MOUNTING EMBEDDED OTOLITHS

Consumables
cyanoacrylate ester glue (eg. Super-Glue)

The embedded otoliths are aligned and attached to the glass slide for sectioning. Place one very small drop of glue on the scored area of the slide. Place "bullet" on glue drop, aligning embedded otolith so that the desired axis of cut is parallel to the scored lines and so that the primordia is centered between them. To overcome parallex problems, align the slide under the microscope so that the scored lines are horizontal. Allow glue to dry at least 10 minutes.

SECTIONING OTOLITHS

Consumables
dishwashing detergent

Equipment
low speed saw
2 diamond wafer blades
blade spacer(s)
stereo microscope head equipped with ocular micrometer, and mounted over saw blades (Fig. 1)

Otoliths are sectioned to yield a relatively thin section with parallel sides encompassing the primordia. Sectioning also gives the option of selecting the plane of cut.

Follow the directions under SCORING SLIDES to prepare the saw. Place the glass slide with mounted otolith in the chuck (Fig. 2). Use the microscope to align the otolith. Adjust the slide until the scored lines are parallel to the saw blades. Move the saw arm until the score marks are approximately over the blades. Focus to the plane of the blade edges. Note their position with the ocular micrometer. Focus on the scores. Adjust slide so that scores appear one full ocular micrometer unit closer to the saw motor than are the saw blades. (They are not actually closer, but we are accounting for the optical distortion due to the thickness of the resin). Add 50 g weight to arm, start saw and section until glass slide reaches the blades.

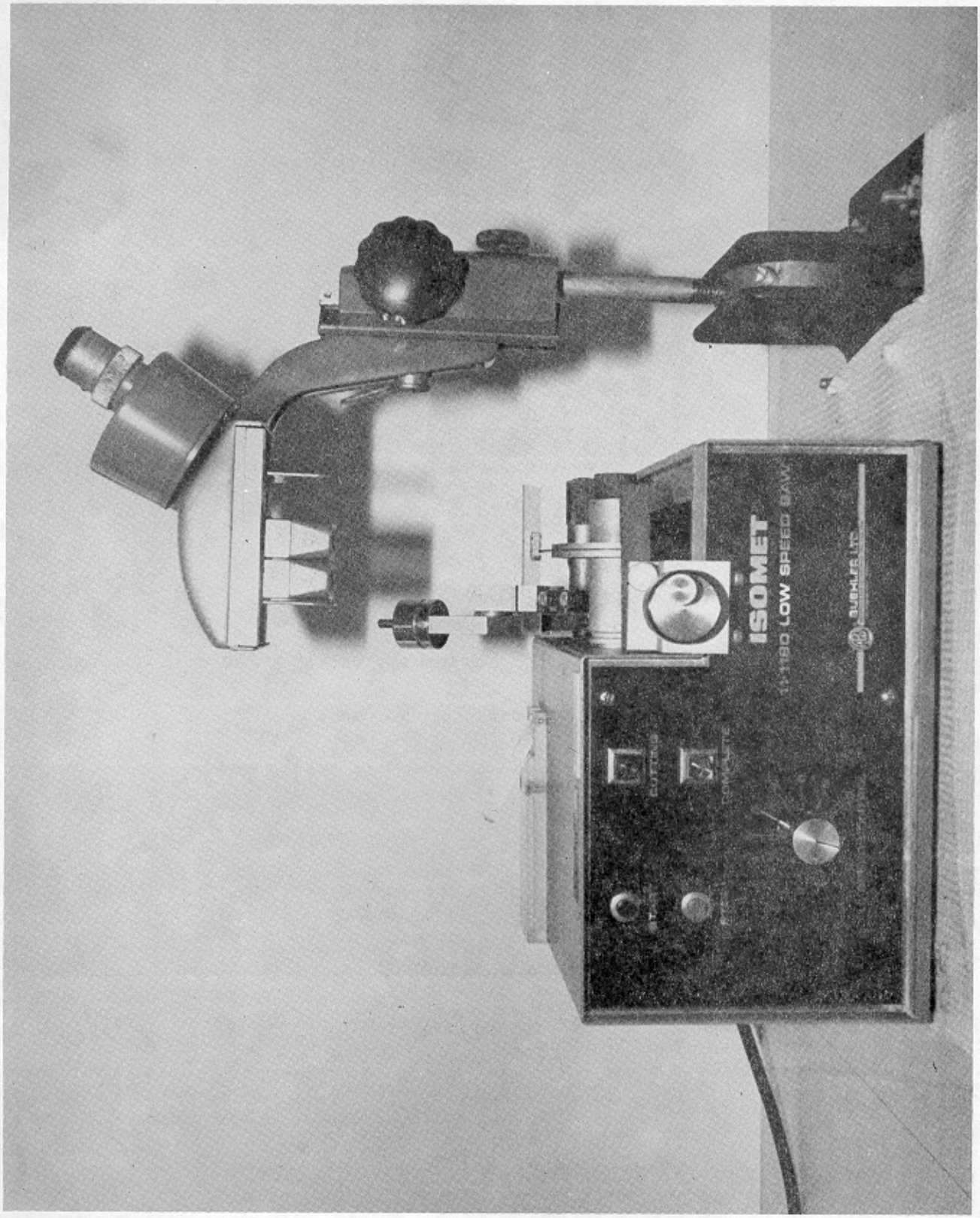

Figure 1. Hardware used to section otoliths.

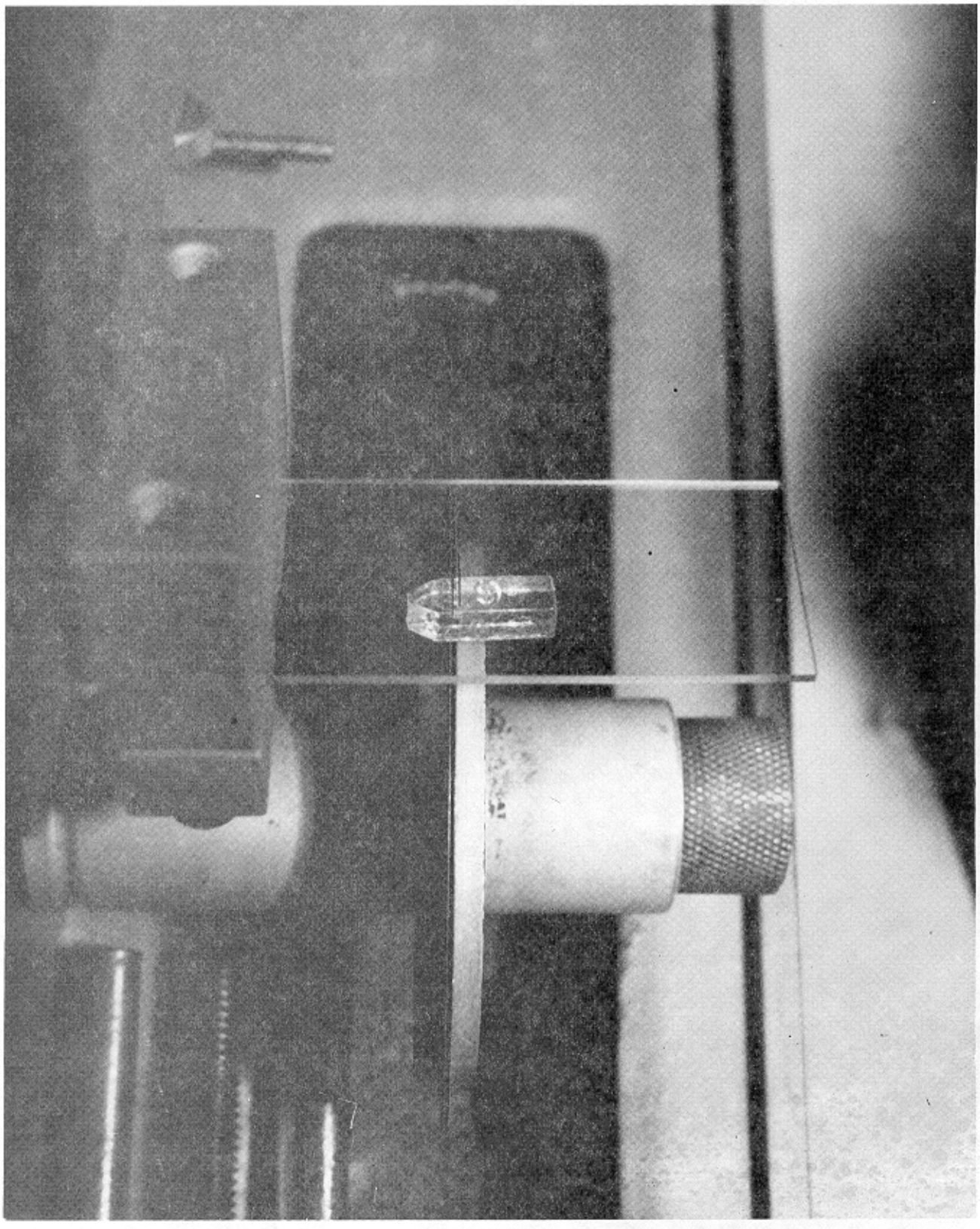

Figure 2. Aligning slide containing embedded otolith in the saw.

MOUNTING SECTIONS

Consumables	Equipment
single-edge razor blade	hot plate
ink marking pen	
25 x 75 mm microscope slides	
thermoplastic resin	

Otolith sections are mounted on glass slides for further processing and for reading.

Examine sectioned "bullet" under microscope and determine which side has the greatest distance between the otolith's primordia and the surface of the cut. Mark that side. With a single-edge razor blade separate the section from the remainder of the "bullet".

Place the slide on a hot plate. When slide is hot, touch a stick of thermoplastic resin to slide. With forceps move the section, oriented with side with the greater distance to the primordia facing up, into the hot cement on the slide. Immediately move the slide from the hot plate and with a fine probe, press air bubbles out from under the section. Allow to cool for at least five minutes.

POLISHING OTOLITH SECTIONS

Consumables	Equipment
abrasive sheets:	glass plates, approx. 100x100 mm
12μ	ultrasonic cleaner
9μ	100 ml beaker
3μ	compound microscope equipped
1μ	with polarized light and at
0.3μ	least a 10x and 40x objective
Immersion oil	hot plate
Photo Flo	

Otolith sections are polished to remove the material overlying the primordia and to remove scratches from sectioning and from previous polishing.

Prepare a sequence of polishing papers by attaching individual papers to flat glass plates. Start with 600 grit (12-14μ) and progress smaller to 9μ, 3μ, 1μ and 0.3μ papers. Place a drop of oil on the section and place the slide, section side down,

on the coarsest paper. With light pressure and circular motions polish the surface, stopping frequently to view the section under the microscope with polarized light. Focus through the primordia to determine how quickly you are approaching it. Throughout the polishing steps maintain the microscope slide parallel to the underlying glass plate and keep the papers well lubricated. Wipe section clean after polishing each time. When the primordia is in view, move to a smaller grit paper and polish closer. Polish slowly and keep clean papers on the glass plates.

When switching to a finer grit paper, and before ever using oil immersion objectives, clean the section in the ultrasonic cleaner. Place a couple of drops of 1% Photo Flo solution (or 1 drop of the concentrated product) into a 100 ml beaker of distilled water and place the beaker in the water bath of an ultrasonic cleaner. Wipe the section to remove most of the oil and grit and place the slide in the beaker. Allow section to be cleaned at least 30 seconds.

When one side has been polished to the primordia, clean the slide ultrasonically and if polishing from the opposite side is necessary, place it (section facing up) on a hot plate set to low heat. When the resin has heated sufficiently, use probes to flip the section. Remove slide immediately and press out any air bubbles. Allow to cool. Polish second side to primordia. The section may be flipped more than once, but after several heating and cooling cycles, the resin may lose its ability to soften, so minimize the number of times the section is flipped and its contact with heat. The section is now ready to be read under a light microscope.

PREPARING OTOLITH SECTIONS FOR SEM

Consumables
None

Equipment
ultrasonic cleaner
coverslipping forceps
glass cutter

Sections to be prepared for SEM must be well polished to the primordia using the finest abrasive paper and must be rinsed in acetone and etched. With a glass cutter score a square around the section on the glass slide and break the slide at the scores. The remaining shard containing the section should be approximately the size of the SEM stub. Clean the section in the ultrasonic cleaner; it is very important to have a clean surface free of oils and grit.

RINSING AND ETCHING SECTIONS

Consumables	Equipment
200 ml 0.1N HCl	coverslipping forceps
200 ml 7% disodium EDTA, pH7.5 (adjust pH with solid NaOH)	compound microscope equipped with polarized light
acetone	

It is necessary to rinse the section briefly to remove any polyester resin which may have smeared over the surface of the otolith section during polishing. Etching creates the 3-dimensional surface required for SEM imaging.

Clean the polished section in the ultrasonic cleaner. Holding the glass shard with the forceps, rinse section in acetone for no longer than 5 sec. Rinse with water and etch with either HCl or EDTA. Etching times given are for juvenile Atlantic menhaden and may vary with size or other species. Be careful not to touch the surface of the polished otolith.

HCl: Place the glass shard in the container of 0.1N HCl and etch for 30 seconds. Rinse with water, dry and view with a compound microscope. If more etching is needed, etch for 10 second periods, up to a cumulative etching total of 60 seconds. Rinse and view the section after every 10 second period.

EDTA: Place the glass shard in the container of 7% EDTA held at 30°C and etch for 3 minutes. Rinse with water, dry and view with a compound microscope. If more etching is needed, etch for 30 second periods, up to a cumulative etching total of 5 minutes. Rinse and view the section after every 30 second period.

MOUNTING SPECIMEN ON STUBS AND SPUTTER COATING

Consumables	Equipment
double-sided transparent tape	coverslipping forceps
silver paint	SEM stubs

To view the specimen with the SEM, the specimen must be mounted and coated with an electron-dense material. Electrons from the SEM emitter are bounced off the electron-dense surface and picked up by the SEM to image the specimen. Sputter coating provides a very uniform thin surface. The silver paint, also an electron-dense material, is used to ensure the area around the specimen does not charge (absorb the electrons), interfering with the image.

Dry the specimen with hot air. Without touching the surface of the section, attach the dry glass shard to the SEM stub (section facing up) with double-sided tape. The section must be sputter coated to a thickness of approximately 90 Å. After sputter coating, paint the surface, except for the resin sliver containing the sectioned otolith, with non-flaring silver paint. Allow the paint to dry.

The specimen is now ready to be read with a SEM. When using the SEM, enhanced images are achieved by tilting the specimen and sometimes by enabling the backscatter feature.

VENDORS

We have used these vendors but are not endorsing them as there may be other suppliers. This list is for information only.

Aremco Products, Inc.
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Check# P333774

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Castolite polyester resin, 1lb.	20-8120-001
Castolite hardener, 1 unit	20-8122-001
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long saddle chuck	11-1187
pair of 1 3/4 inch flanges	11-1191
Fibermet abrasive sheets:	
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9μ	61-3151
3μ	61-3152
1μ	61-3153
0.3μ	61-3154

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Tustin, CA 92681
800-237-3526

105 Flat embedding mold 105
Transparent embedding mold 10504
110 Flat embedding mold 110